A novel mouse model of phospholipase A2 receptor 1-associated membranous nephropathy mimics podocyte injury in patients

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The phospholipase A2 receptor 1 (PLA2R1) is the major autoantigen in patients suffering from membranous nephropathy. To date, the lack of endogenous glomerular expression of PLA2R1 in mice and rats has impeded the establishment of PLA2R1-dependent animal models of this disease. Here, we generated a transgenic mouse line expressing murine full-length PLA2R1 in podocytes. Furthermore, expression of murine PLA2R1 did not result in any morphological disturbance as high-resolution confocal microscopy demonstrated an intact nephrin distribution with normal foot processes. Transfer of rabbit anti-mPLA2R1 antibodies to these mice induced nephrotic range proteinuria, hypercholesterolemia, and histomorphological signs of membranous nephropathy. Immunohistochemical and immunofluorescence analyses revealed enhanced staining for murine PLA2R1 in the presence of unaffected staining for murine thrombospondin type-1 domain-containing 7A in the diseased mice, resembling what is classically found in patients with PLA2R1-associated membranous nephropathy. Thus, our mouse model of membranous nephropathy will allow investigation of PLA2R1-specific pathomechanisms and may help to develop and assess antigen-specific treatments in vivo.

Translational Statement

Over the last decade, the measurement of anti-phospholipase A2 receptor 1 (PLA2R1) antibodies for the diagnosis and monitoring of patients with membranous nephropathy (MN) has been broadly implemented into clinical practice worldwide. Despite this major advance, MN pathomechanisms are still incompletely understood, and patient treatment relies on broad and unspecific immunosuppression. We envision that this model of PLA2R1-associated MN may help to dissect the molecular mechanisms that underlie podocyte injury in MN. The roles of the complement system and receptor-specific podocyte signaling programs represent subjects of future interest that can be addressed using this model. Additionally, innovative therapeutic strategies targeting MN-specific pathomechanisms can be investigated regarding their in vivo efficacy—a prerequisite for the translation to patient care in the future.

Membranous nephropathy (MN) is an autoimmune glomerular disease and a frequent cause of nephrotic syndrome. Morphologically, the disease is characterized by granular deposition of IgG and complement system components along the glomerular filtration barrier as well as by subepithelial electron-dense deposits and podocyte foot process effacement in electron microscopy.1,2 Circulating autoantibodies against the podocyte membrane proteins phospholipase A2 receptor 1 (PLA2R1) and thrombospondin type-1 domain-containing 7A (THSD7A) are found in around 75%–80% of cases.3,4 Although recent animal studies demonstrated a pathogenic role of anti-THSD7A antibodies in the development of MN,5,6 the lack of endogenous PLA2R1 expression in rodents’ precluded analogous antibody transfer studies. However, as the majority of MN cases are PLA2R1-associated, the development of a mouse model involving this antigen is fundamental in order to experimentally investigate the role of anti-PLA2R1 antibodies, understand the pathogenic route through which anti-PLA2R1 autoantibodies induce...
disease, and develop and evaluate novel antigen-specific treatments.

RESULTS

Generation and basal characterization of mouse PLA2R1 (mPLA2R1)-positive mice

Mice expressing mPLA2R1 in podocytes (mPLA2R1-positive mice) and their littermate controls (mPLA2R1-negative mice) were generated using a transgenic knock-in approach (Supplementary Methods and Supplementary Figure S1). The transgenic mPLA2R1 protein was strongly expressed in the podocyte membrane and cytoplasm of mPLA2R1-positive mice, whereas mPLA2R1 expression was completely absent in podocytes of mPLA2R1-negative mice (Figure 1a). In mPLA2R1-positive mice, mPLA2R1 expression partially merged with the slit diaphragm protein nephrin, indicating a specific localization at podocyte foot processes. Expression of mPLA2R1 did not result in a morphologic disturbance of foot process morphology, as high-resolution confocal microscopy demonstrated an intact nephrin distribution with normal foot process morphology (Figure 1b). Electron microscopy confirmed a regular foot process morphology and demonstrated normal glomerular basement membranes and endothelium in all mice (Figure 1c). Additionally, light microscopy showed no alterations in overall glomerular and tubulointerstitial morphology (Figure 1d). The number of podocytes identified by p57 staining was also similar in the 2 groups (Figure 1e). Measurement of the albumin/creatinine ratio in the urine of these mice excluded an mPLA2R1-mediated altered permeability of the glomerular filtration barrier to protein, determined over the course of 15 weeks (Figure 1f). Finally, we found no activation of pathways indicating cellular stress (Supplementary Figure S2), excluding an overload of cellular degradation/repair systems related to the transgenic expression of mPLA2R1. Together, these results demonstrate the successful generation of an mPLA2R1-expressing mouse line.

Generation of rabbit anti-mPLA2R1 antibodies

Rabbit anti-mPLA2R1 antibodies were generated by means of cDNA immunization. Incubation of Chinese hamster ovary (CHO) cells cotransfected with mPLA2R1 and green fluorescent protein with rabbit antiserum, but not with serum taken before immunization, resulted in a specific fluorescent signal for membrane-bound rabbit IgG on those cells showing a nuclear green fluorescent protein signal, demonstrating the presence of mPLA2R1-specific serum antibodies (Supplementary Figure S3A). Total IgG purified from the rabbit serum after immunization (referred to as anti-mPLA2R1 IgG) showed strong and specific binding to mPLA2R1 present in mouse glomerular extracts purified from mPLA2R1-positive mice under nonreducing conditions, and a complete absence of binding when mPLA2R1 was reduced (Figure 2a). This finding is in accordance with the known characteristics of patient anti-PLA2R1 autoantibodies, which also recognize conformation-dependent epitopes within the target antigen. Of note, a faint signal could also be detected in glomerular extracts from mPLA2R1-negative mice (and wild-type BALB/c mice, data not shown), possibly indicating a low intrinsic expression of mPLA2R1 in mouse glomeruli. The anti-mPLA2R1 IgG strongly recognized the N-terminal region of mPLA2R1 comprising the cysteine-rich domain, the fibronectin type II domain, and the first C-type lectin domain (CysR-CTLD1), and bound additional epitopes in the CTLD1-2, CTLD2-6, and CTLD2-8 regions, but not in the CTLD7-8 region (Supplementary Figure S3B).

Disease development after transfer of anti-mPLA2R1 IgG

We next injected anti-mPLA2R1 IgG (total IgG purified from mPLA2R1-immunized rabbits) or control IgG (total IgG purified from non-immunized rabbits) intraperitoneally into mPLA2R1-positive and/or mPLA2R1-negative mice. mPLA2R1-positive mice injected with anti-mPLA2R1 IgG, but not the other groups, rapidly developed proteinuria up to an albumin/creatinine ratio of 100 g/g that was sustained over the complete observation period of 7 days (Figure 2b). Urinary samples of these mice contained, in addition to albumin as the most prominent protein at 69 kDa, both low (below 69 kDa) and high (above 69 kDa) molecular weight proteins (Supplementary Figure S3C), indicating a major perturbation of the glomerular filtration barrier. When the amount of injected rabbit IgG was reduced, proteinuria also decreased, suggesting a dose-dependent effect (data not shown). We also analyzed proteinuria up to 21 days (during the autologous phase of the disease) in an independent experimental cohort and found that albumin-to-creatinine ratios slowly decreased below 100 g/g (data not shown).

In addition, serum cholesterol levels were significantly increased in mPLA2R1-positive mice receiving anti-mPLA2R1 positive rabbit IgG (Figure 2c), whereas serum urea nitrogen levels remained normal (Figure 2d). Taken together, these results demonstrate that anti-mPLA2R1 antibodies cause nephrotic-range proteinuria and hypercholesterolemia in mPLA2R1-positive mice.

Histologic and ultrastructural changes after transfer of anti-mPLA2R1 IgG

We next investigated the histologic changes in mice after transfer of anti-mPLA2R1 IgG. We found granular and subepithelial deposition of rabbit IgG in mPLA2R1-positive mice 5 and 7 days after injection of anti-mPLA2R1 IgG (Figure 2e; Supplementary Figure S4). In contrast, rabbit IgG staining along the glomerular filtration barrier was absent in mPLA2R1-positive mice treated with control IgG, as well as mPLA2R1-negative mice treated with anti-mPLA2R1 IgG (Figure 2e; Supplementary Figure S5), albeit the latter showed very faint positivity for rabbit IgG in the mesangial space, again possibly indicating some intrinsic mPLA2R1 expression in the glomeruli of these mice. However, attempts to localize intrinsic glomerular mPLA2R1 expression in wild-type and in mPLA2R1-negative mice by immunohistologic techniques were unavailing. Notably, mPLA2R1-positive mice treated with anti-mPLA2R1 IgG showed positivity for complement
C3 in partial colocalization with the bound rabbit IgG at day 7, suggesting complement activation in the area of immune complex deposition (Figure 2f). This activation of the complement system was accompanied by oxidative stress as indicated by an upregulation of superoxide dismutase 1 (SOD1) in podocytes (Supplementary Figure S6).

Antibody elution experiments from renal cryosections derived from mPLA2R1-positive mice 7 days after injection of mPLA2R1-negative mice backcrossed to the BALB/c background in comparison to mPLA2R1-negative control littermates was evaluated. (a) Confocal microscopy shows the expression of mPLA2R1 (green) along the glomerular filtration barrier in close proximity to the slit diaphragm protein nephrin (red) and in the cytoplasm of podocytes exclusively in mPLA2R1-positive mice. Blue, nuclei. (b) High-resolution confocal microscopy staining for nephrin demonstrates regular foot process (FP) morphology in mPLA2R1-positive mice. Blue, nuclei. (c) Electron micrographs of the glomerular filtration barrier exhibit normal architecture of the endothelial cell layer, the glomerular basement membrane (GBM), and podocytes in mPLA2R1-positive mice. (d) Periodic acid–Schiff staining shows normal glomerular and tubulointerstitial morphology in mPLA2R1-positive mice. (e) The number of podocytes identified by p57 staining (green) was comparable between mPLA2R1-positive and -negative animals. Red, nephrin; blue, nuclei. (f) Albumin/creatinine ratios as measured by enzyme-linked immunosorbent assay from 7 up to 15 weeks of age. Values are expressed as mean ± SEM; n = 7 for mPLA2R1-negative mice; n = 11 for mPLA2R1-positive mice. C, capillary; U, urine. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.
Figure 2 | Passive transfer of anti-mouse phospholipase A2 receptor 1 (mPLA2R1) IgG induces membranous nephropathy in mPLA2R1-positive mice. (a) Western blot analyses of mouse glomerular (glom.) extracts with anti-mPLA2R1 IgG under nonreducing and reducing conditions. Note the specific reactivity with mPLA2R1 at 180 kDa solely under nonreducing conditions. Anti-beta actin detection was used with the same membrane as a loading control. (b) Time-course of albuminuria as measured by albumin/creatinine ratio. Values are indicated as mean ± SEM following the application of 3 milligrams of anti-mPLA2R1 IgG or control IgG to mPLA2R1-positive mice (continued)
either control or anti-mPLA2R1 IgG demonstrated specific binding of eluted antibodies to both recombinant mPLA2R1 and mPLA2R1 present in mouse glomerular extracts (Figure 2g). In periodic acid–Schiff stainings, mPLA2R1-positive mice treated with anti-mPLA2R1 IgG exhibited protein casts in dilated tubuli, reflecting the heavy proteinuria (Supplementary Figure S7A). Immunohistochemical and immunofluorescence analyses revealed enhanced staining for mPLA2R1 in the presence of unaffected staining for mouse THSD7A in the diseased mice (Supplementary Figure S7B–D), resembling the situation that is classically found in patients with PLA2R1-associated MN.4,5 In electron microscopic analyses, we found electron-dense deposits in a strictly subepithelial location within the glomerular basement membrane and areas of foot process broadening in mPLA2R1-positive mice injected with anti-mPLA2R1–positive IgG, but not in control mice (Figure 2h; Supplementary Figure S8). Of note, electron-dense deposits were found in close proximity to the podocyte slit diaphragms. In line, high-resolution confocal imaging revealed areas of foot process effacement and colocalization of mPLA2R1 and the deposited rabbit IgG with the slit-diaphragm protein nephrin (Supplementary Figure S9A), suggesting, as described previously for experimental THSD7A-associated MN,6 that the slit diaphragm is an early target of mPLA2R1-anti-mPLA2R1 antibody immune complex formation. Additionally, disruption of nephrin architecture indicated slit diaphragm alterations induced by the anti-PLA2R1 IgG (Supplementary Figure S9B).

Taken together, these results demonstrate that mPLA2R1-expressing mice develop the typical histomorphologic signs of human MN after transfer of anti-mPLA2R1 antibodies.

DISCUSSION
The purpose of this study was the establishment and characterization of a murine model of PLA2R1-associated MN. In this regard, we (i) developed a transgenic mouse line expressing mPLA2R1 in podocytes, (ii) generated mPLA2R1-specific antibodies in rabbits, and (iii) found that transfer of these antibodies causes MN in PLA2R1-expressing mice. Thus, the clinical and histologic features of MN could be successfully reproduced in mice, representing an antigen-specific passive immunization model of PLA2R1-associated MN.

Previous attempts of our group to induce MN in mice expressing human PLA2R1 fused to a glycosylphosphatidylinositol anchor were unsuccessful (Zahner G, Helmchen U, Stahl RA. The generation of inducible specific human phospholipase A2 receptor transgenic mice [abstract]. J Am Soc Nephrol. 2012;23:568A; Zahner G, Tomas N, Hoxha E, et al. Development and morphologic characterization of a mouse model of membranous nephropathy involving the human phospholipase A2 receptor [abstract]. J Am Soc Nephrol. 2014;25:66A). This lack of success could be related to the complex structure of PLA2R1,10 resulting in insufficient antigen expression and membrane incorporation of the human protein in mice. In an attempt to circumvent these issues, we decided to transgenically express the murine PLA2R1 orthologue (mPLA2R1), which shares a moderate amino acid identity of 72% with human PLA2R1. We also injected a few mPLA2R1-positive mice with patient-derived anti-PLA2R1 IgG, but we could hardly detect any glomerular binding of human IgG, and the mice did not develop proteinuria. In Western blot analysis, we found a markedly reduced binding of patient serum with recombinant mPLA2R1, indicating that the human anti-PLA2R1 autoantibodies cannot bind the murine antigen to an extent sufficient to induce disease. This lack of disease development when using patient autoantibodies is a limitation of our model.

Application of this PLA2R1-specific MN model will help to address several important questions in the field of MN.11,12 Studies in the Heymann nephritis model indicated that local activation of the complement system with subsequent podocyte damage by the membrane attack complex C5b–9 is the key step in induction of proteinuria in MN.13,14 More recent studies suggest additional complement-independent mechanisms of antibody pathogenicity in MN, such as disturbance of cell adhesion to collagen type IV by anti-PLA2R1 antibodies,15 cytoskeletal rearrangement and alterations in focal adhesion signaling induced by anti-THSD7A antibodies,7 and inhibition of target antigen enzymatic activity by anti–neutral endopeptidase antibodies.16,17 Notably, we found some glomerular deposition of complement C3 in...
our mouse model of PLA2R1-associated MN, in contrast to results in the recently published mouse model of THSD7A-associated MN, in which C3 was barely detectable. Future studies are needed to investigate the pathogenic relevance of glomerular complement activation and antibody-induced alterations in antigen function and podocyte signaling in experimental PLA2R1- and THSD7A-associated MN.

The identification of the antibody binding regions in PLA2R1 and THSD7A will give rise to the development of novel and individualized therapies. For example, antibody clearance using epitope-specific immunoadsorption, trapping of pathogenic antibodies by antibody-binding fragments, and epitope blockage using specifically engineered antagonists represent therapeutic approaches that can be evaluated for their in vivo efficacy in this experimental passive model of PLA2R1-associated MN. The preclinical testing of innovative treatments targeting the autoantibody-producing cells, however, would require an animal model involving the production of autoantibodies by members of the B-cell lineage. Our mPLA2R1-expressing mice may enable the generation of such an active immunization model by means of immunization with either the mPLA2R1 protein or mPLA2R1 cDNA. Interestingly, recognition of epitopes in PLA2R1 beyond the cysteine-rich domain has been found to be associated with higher proteinuria, poor clinical outcome during follow-up, and reduced response to treatment with rituximab in patients with MN. However, it is unclear whether this diversified antigen recognition results from differential autoantibody repertoires already defined at disease initiation or from intramolecular epitope spreading over time. In Heymann nephritis, epitope spreading from the N-terminal toward the C-terminal region occurred when rats were immunized with a 236-mer N-terminal residue of megalin. Whether this immunologic phenomenon is also part of the immune response against PLA2R1 could be investigated in such an active disease model. Furthermore, how antibody binding to single or multiple epitopes relates to disease severity can be addressed using the herein described passive model of PLA2R1-associated MN by means of domain-specific antibodies.

In conclusion, we generated a murine PLA2R1-dependent MN mouse model that can be robustly induced by rabbit anti-PLA2R1 antibodies and replicates human PLA2R1-associated MN. This model opens new avenues to comprehensive understanding of the molecular mechanisms that underlie podocyte injury in PLA2R1-associated MN and to investigation of innovative therapeutic strategies targeting MN-specific pathomechanisms regarding their in vivo efficacy—a prerequisite for translation to patient care in the future.

METHODS

Details on the transgenic generation of mPLA2R1-positive mice and rabbit anti-mPLA2R1 antibodies, antibody purification, construction of animal experiments, histologic analyses, electron microscopy studies, cell culture, isolation of mouse glomeruli, antibody elution, and statistical analyses are presented in the Supplementary Methods.

DISCLOSURE

NMT holds a patent: “Methods and Kits for Monitoring Membranous Nephropathy.” All the other authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods and Supplementary References.

Figure S1. Generation of mPLA2R1 full-length podocyte-specific transgenic mice.

Figure S2. Transgenic expression of mPLA2R1 does not result in cellular/proteostatic stress.

Figure S3. Generation of anti-PLA2R1 antibodies in rabbits and antibody transfer.

Figure S4. mPLA2R1-positive mice treated with rabbit anti-mPLA2R1 antibodies but not with control rabbit IgG deposit rabbit IgG at the glomerular filtration barrier on day 5.

Figure S5. mPLA2R1-negative mice with rabbit anti-mPLA2R1 antibodies do not deposit rabbit IgG nor complement at the glomerular filtration barrier in comparison to mPLA2R1-positive mice.

Figure S6. Superoxide dismutase 1 (SOD1) is upregulated in mPLA2R1-positive mice treated with rabbit anti-mPLA2R1 antibodies.

Figure S7. Light microscopic changes and mPLA2R1 staining in mPLA2R1-positive mice 7 days after the transfer of control IgG and anti-mPLA2R1 IgG.

Figure S8. Electron microscopy studies.

Figure S9. High-resolution confocal and electron microscopic evaluations of immune complexes 7 days after the injection of rabbit anti-mPLA2R1 IgG to mPLA2R1-positive mice.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES


